

SALICYLIC ACID COLLABORATES WITH GENE SILENCING TO TOMATO DEFENSE AGAINST TOMATO YELLOW LEAF CURL VIRUS (TYLCV)

YUNZHOU LI^{1,2}, TAYEB MUHAMMAD¹, YONG WANG², DALONG ZHANG³,
M. JAMES C. CRABBE^{4,5}, AND YAN LIANG^{1*}

¹College of Horticulture, Northwest A&F University, Yangling 712100, Shaanxi, China;

²College of Agriculture, Guizhou University, Guiyang, 550025, Guizhou, China;

³College of Horticultural Science and Engineering, Shangdong Agricultural University, Tai'an, Shandong, China;

⁴Department of Zoology, University of Oxford, The Tinbergen Building, South Parks Road, Oxford, UK;

⁵Institute for Biomedical and Environmental Science and Technology, Department of Life Sciences, University of Bedfordshire, Park Square, Luton, UK

*Corresponding author's e-mail: liangyan@nwsuaf.edu.cn

Abstract

Antiviral research in plants has been focused on RNA silencing (i.e. RNA interference), and several studies suggest that salicylic acid (SA)-mediated resistance is a key part of plant antiviral defense. However, the antiviral defense mechanism of SA-mediation is still unclear, and several recent studies have suggested a connection between SA-mediated defense and RNA silencing, which needs further characterization in TYLCV infection. In this study, both SA-mediated defense and the RNA silencing mechanism were observed to play an important role in the antiviral response against TYLCV. First, we found that SA application enhanced the resistance to TYLCV in tomato plants. The expression of RNA-silencing-related genes, such as *SIDCL1*, *SIDCL2*, *SIDCL4*, *SIRDR2*, *SIRDR3a*, *SIRDR6a*, *SIAGO1*, and *SIAGO4*, were significantly triggered by exogenous SA application and inoculation with TYLCV, respectively. Furthermore, silencing of *SIDCL2*, *SIDCL4* in tomato resulted in attenuated resistance to TYLCV, and reduced the expression of defense-related genes (*SIPR1* and *SIPR1b*) in SA-mediated defense after infection with TYLCV, particularly in *SIDCL2/SIDCL4*-silenced plants. Taken together, we conclude that SA collaborates with gene silencing in tomato defense against TYLCV.

Key words: Salicylic acid, Gene silencing, Tomato Yellow Leaf Curl Virus, defense

Introduction

Plants are threatened with a variety of abiotic and biotic stresses; of the latter, viruses are the most serious pathogens (Scholthof *et al.*, 2011). To survive, plants have evolved sophisticated defense mechanisms against viral infection. Multiple antiviral mechanisms demonstrate means of limiting virus replication and movement (Incarbone & Dunoyer, 2013; Faoro & Gozzo, 2015). Much research has been focused on RNA silencing, a sequence-specific RNA degradation mechanism in higher eukaryotes, which encompasses post-transcriptional gene silencing (PTGS) and RNA interference (RNAi). RNA silencing regulates or mediates the expression levels of endogenous and exogenous genes even viruses, transgenes and transposable elements (Ma *et al.*, 2015). This defense mechanism has many essential players including Dicer-like (DCL) proteins, Argonaute (AGO) proteins, and RNA-dependent RNA polymerase (RDR) (Manni *et al.*, 2015). In plants, RNA silencing defense against viruses depend on DCL2, DCL3 and DCL4 enzymes to recognize viral double-stranded RNA (dsRNA) and process it into virus-derived small interfering RNAs (vsiRNA) of 21-24 nt (nucleotide). In *Arabidopsis thaliana* plants, immunity against viruses is provided by *DCL4*, *DCL2*, and *DCL3*, which target the viral genomes and produce vsiRNAs of 21, 22, and 24 nt, respectively (Axtell, 2013). DCL2 and DCL4 have been suggested as two key components in RNAi responses against RNA viruses (Deleris *et al.*, 2006; Qu *et al.*, 2008; Moissiard *et al.*, 2016).

Salicylic acid (SA) is a naturally synthesized phenolic compound that is essential for the establishment of local and systemic resistance in plants against a wide range of pathogens (Vlot *et al.*, 2009). Exogenous SA application induces systemic acquired resistance (SAR) and triggers the synthesis of pathogenesis-related (PR) protein groups, which enhance activation of a variety of defense responses against major pathogens in plants (El-Shetehy *et al.*, 2014). In addition, salicylate hydroxylase (*nahG*) plants, where a bacterial transgene encoding NahG has been introduced, are also more susceptible to pathogens because of absent or reduced SA accumulation (Benouaret & Goupil, 2015). Previous studies have focused on the function of antimicrobial defense (Vlot *et al.*, 2009). Recently, studies have begun to focus on the role of SA in antiviral defense, such as in Tobacco mosaic virus (TMV), Cucumber mosaic virus (CMV), Turnip crinkle virus (TCV) and Tobacco necrosis virus (TNV) (Vlot *et al.*, 2009; Shang *et al.*, 2011). Similarly, it was observed that SA or gentisic acid (GA, a metabolic derivative of SA) application induced RNA silencing-related genes to delayed RNA virus and viroids accumulation (Campos *et al.*, 2014). However, most studies have been concerned with RNA-based pathogens, and the mechanism of SA-antiviral defense is still not clear.

Tomato yellow leaf curl virus (TYLCV) is a single-stranded DNA virus that belongs to the genus *Begomovirus* and family *Geminiviridae*. This virus is transferred from infected plants to healthy plants by the whitefly (*Bemisia tabaci*) and causes tomato yellow leaf curl disease (TYLCD) (Czosnek, 2007). Five TYLCV resistance/tolerance genes (*Ty-1*, *Ty-2*, *Ty-3*, *Ty-4* and *Ty-5*) have been mapped and identified (Ji *et al.*, 2009;

Hutton *et al.*, 2012; Verlaan *et al.*, 2013; Yang *et al.*, 2014). Recent cloning of *Ty-1* and *Ty-3* suggest they are allelic (Verlaan *et al.*, 2013). Both *Ty-1* and *Ty-3* encode RNA-dependent RNA polymerase (RDR) belonging to the RDR γ type, which may be involved in RNA silencing.

Gene silencing and SA-mediated defense belongs to induced resistance which occurs in plants infected with pathogens. RNA silencing consists of a series of interconnected pathways that limit the synthesis, stability and translatability of foreign or aberrant RNAs. In the first stage (a), the presence or formation of dsRNA needs an RNA-dependent RNA polymerase (RDR or RdRP). In the second stage (b), Dicer-like endoribonucleases (*DCL*) process dsRNA into small RNA fragments called siRNA, which (c) are incorporated into a complex that is associated specifically with the complementary RNA target (Carr *et al.*, 2010). *DCL2* and *DCL4* have been reported as two key components in RNAi responses against incompatible RNA viruses (Deleris *et al.*, 2006; Qu *et al.*, 2008; Campos *et al.*, 2014). *DCL2* and *DCL4* are required for “Primary” short-interfering (siRNA), which is amplified to produce “secondary siRNA” by RDR polymerases (Moissiard *et al.*, 2016). However, little is known about the function of *DCL2* and *DCL4* in tomato defense against compatible DNA virus.

Several studies suggest a connection between SA-mediated defense and RNA silencing (Baebler *et al.*, 2014; Campos *et al.*, 2014; Lee *et al.*, 2016). For example, the expression levels of tomato *DCL1*, *DCL2*, and *RDR2*, were induced by exogenous SA application (Campos *et al.*, 2014).

The objectives of this study were to clarify the role of *SIDCL2* and *SIDCL4* in compatible virus, such as that linked to TYLCV, and the relationship of RNA silencing with SA-mediated defense through a TYLCV-tomato pathogens interaction system. Here, we firstly confirmed that the SA-mediated mechanism participated in antiviral defense by exogenous application of SA. That RNA silencing also participates in antiviral activity was confirmed by silencing two tomato genes *SIDCL2* and *SIDCL4* using virus-induced gene silencing (VIGS) techniques. We found the two defense related genes (*SIPRI* and *SIPRIb*) in the SA-defense pathway to be down-regulated in *SIDCL2*-, *SIDCL4*-, and *SIDCL2/4*- silenced plants, particularly in the *SIDCL2/4*- silenced plants. We argue that the function of *DCL2* and *DCL4* in antiviral defense and its relationship with SA-mediated defense pathways can be identified through virus-host interactions with TYLCV in tomato. Silencing of *SIDCL2* and *SIDCL4* in tomato decreased resistance to TYLCV, and this process might be collaborated with SA-mediated pathways.

Materials and Methods

Plant materials: Tomato (*Solanum lycopersicum*) ‘TTI112B-2’ and ‘Y19’ seeds were obtained from the Tomato Germ Plasm Resource Lab, College of Horticulture, Northwest A&F University, Yangling, China. The ‘TTI112B-2’ is a susceptible and ‘Y19’ is a resistant material having *TY-1* and *TY-3* resistance markers (Li *et al.*, 2017). Seeds were planted in pots with a 30cm diameter containing a 1:1 mixture of peat and

vermiculite. Plants were grown at $25 \pm 3^\circ\text{C}$ with 50-70% relative humidity under 16 hours photoperiods in an insect free greenhouse. For the VIGS experiment the temperature was kept at $22 \pm 3^\circ\text{C}$ with other conditions as above. Each experiment was replicated three times with 10-15 plants per replication.

SA application and TYLCV inoculation: To confirm SA-induced resistance against TYLCV, tomato seedlings with five true leaves were treated by foliar application of SA (0.5 mM) or buffer alone (50 mM phosphate buffer, pH 7.2). After 48 h (Campos *et al.*, 2014), the plants were inoculated with an infectious TYLCV clone (TYLCV-(CN: SH2) belonging to TYLCV-Israel strains and accession number: AM282874) via *Agrobacterium tumefaciens*-mediated inoculation. The clone was infiltrated ($\text{OD}_{600}=1.5$) into the phloem of plant stems at three different points as previously described (Bai *et al.*, 2012). Virus infection was visually observed and confirmed by PCR as previously described (Eybishtz *et al.*, 2010). Further experiments of disease incidence and disease index were done when inoculated plants reached the sixth- to seventh-true-leaf stage. The control plants were inoculated with *A. tumefaciens* with empty plasmid in a similar manner. The percentage of plant exhibiting disease symptoms (%) and the disease index were determined at 30 days post inoculation (dpi). The calculation formulas of disease incidence (%) and disease index were as follows: Disease incidence = Number of plants with disease symptom/Number of all tested plants $\times 100\%$. Disease index = $[\sum (\text{Number of plants in a scale} \times \text{Corresponding scale value}) / (\text{Total number of plants} \times \text{Highest scale value})] \times 100$ (Li *et al.*, 2017).

DNA and RNA extraction: The third and fourth leaves from the apex were collected from at least three plants 14 days after TYLCV inoculation and immediately frozen in liquid nitrogen. Total DNA was extracted from collected the third and fourth leaves from the apex using the cetyltrimethyl-ammonium bromide (CTAB) method (Fulton *et al.*, 1995) for later detection of TYLCV.

For the gene silencing experiments involving induction by SA, samples were collected at different times after SA treatment: 0, 3, 6, 12, 24, 48, and 72 h. At these times, Total RNA was extracted from each sample using TRIzol (Invitrogen, Carlsbad, CA, USA) and genomic DNA was eliminated by treating the sample with DNase I (Fermentas, Glen Burnie, MD, USA).

In gene silencing experiments involving induction by TYLCV, samples were collected from systemic leaves with disease symptoms (leaf yellowing and curling) as described (Bai *et al.*, 2012) and leaves without symptoms from empty plasmid infiltrated control plants. For SA induction-defense genes (*SIPRI*) experiments, samples were collected at 48 h after SA treatment (Campos *et al.*, 2014).

Quantitative PCR and semi-quantitative PCR: Quantitative PCR (qPCR) was used to detect the relative amount of RNA-silencing-related genes and TYLCV. Total RNA was isolated from leaves using TRIzol (Invitrogen) and genomic DNA was eliminated by treating the samples with DNase I (Fermentas). After

purification, 1.0 µg of total RNA was converted into cDNA by 200 U of Super Script III Reverse Transcriptase (Takara, Dalian, China) in a total reaction volume of 10 µL. The resulting cDNA was diluted to 200 ng/µL and then used for real-time qPCR (qRT-PCR). The qRT-PCR reaction was performed using SYBR Premix Ex Taq II (Takara) and the iQ5 Real-Time PCR Detection System (BIO-RAD Corp., Hercules, California, USA). RNA-silencing-related genes were detected by qPCR using gene-specific primers listed in Supplemental Table 1. *SIEF* was used as a control as previously described (Li *et al.*, 2013; Li *et al.*, 2017). PCR was carried out in 20 µL volume containing 2 µL of diluted cDNA, 250 nM forward primer, 250 nM reverse primer, and 1× SYBR PremixExTaqII (TakaRa Bio, Otsu, Japan). Thermal cycling conditions were pre-denaturation at 95°C for 60 s, followed by 40 cycles of amplification at 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s. Gene relative expression were analyzed using the comparative Ct method (the $2^{-\Delta\Delta Ct}$ method). The procedures and methods used to detect TYLCV via qPCR were similar to those used to detect RNA-silencing-related genes, except the template and primers differed according to methods described previously (Sade *et al.*, 2014).

Semi-quantitative PCR was used to detect TYLCV content as described previously (Eybishtz *et al.*, 2010). For semi-quantitative PCR, a 420-bp fragment corresponding to TYLCV nucleotides 474 to 834 (Gene Bank accession number X15656) was amplified using the primer pair TY-F and TY-R (Supplemental Table 1), and actin (TC198350) was used as a control gene. The PCR reaction mixture comprised of 12.5 µL of 2× Power Taq PCR Master Mix (QIAGEN, Beijing, China), 1.0 µL of each primer (10 µM), 6.5 µL of distilled de-ionized water, and 4 µL of DNA template with a concentration of approximately 70 ng/µL for a total reaction volume of 25 µL. Thermal cycling was performed with an initial denaturation for 3 min at 95°C followed by 28 cycles consisting of 30 s at 94°C, at 54°C for 30 s, and finally 40 s at 72°C. After amplification, 10 µL of the PCR products was subjected to electrophoresis in a 1.2% agarose gel in Tris-acetic acid-EDTA buffer (TAE) and subsequently stained with ethidium bromide.

TRV-based VIGS in *S. lycopersicum*: The online VIGS tool (<http://solgenomics.net/solpeople/login.pl>) was used to target specific genes, and primers were designed using Vector NTI 11.5 (Thermo Fisher Scientific, Waltham, MA, USA). Using the primers described in Supplemental Table 1, fragments of tomato *SIDCL2* or *SIDCL4* were PCR amplified and ligated to the pMD-18T vector and then inserted into the pTRV2 plasmid following digestion by *XbaI/KpnI*. The recombined vector was transferred into *Escherichia coli* (strain DH5α) by heat-shock method, and into *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method. These transformed *Agrobacterium* strains were used in the VIGS experiment. Equal volumes ($OD_{600}=1.0$) of *A. tumefaciens* containing the empty and recombinant plasmid was infiltrated into the first true leaves of three-week-old plants using a previously described method (Liu *et al.*, 2002), and VIGS-phytoene desaturase (*PDS*) was used as a marker of

VIGS silencing in plants (Li *et al.*, 2013; Li *et al.*, 2017). Then, qPCR was used to determine silencing efficiency and specificity of *SIDCL2* or *SIDCL4* compared to other *SIDCL* genes and to analyze the transcript levels of these two genes and other genes in the *SIDCL* family. Results confirming gene silencing as well as the transcript levels of non-targeted genes are presented in Supplemental Fig. 2. Plants were inoculated with TYLCV two weeks after Tobacco rattle virus (TRV) Agro infiltration. Samples were collected 4 weeks after TRV infiltration and 2 weeks after TYLCV inoculation for further analysis. The four treatments in the VIGS experiment were as follows: TRV empty plasmid (TRV: 00), TRV: *SIDCL2*, TRV: *SIDCL4*, and TRV: *SIDCL2/4*.

Statistical analysis

Analysis of variance (ANOVA) was conducted using SPSS version 12.0 software. The significance of differences between means was determined by *t*-test. Data are presented as means ± standard error (SE). Double asterisks and single asterisks indicate significant differences relative to controls at $p<0.01$ and $p<0.05$, respectively. Different letters indicate significant differences compared to control at $p<0.05$.

Results

SA induced resistance to TYLCV in tomato: To study the effect of SA on resistance to the compatible virus, tomato plants were sprayed with SA or buffer solutions. After 2 days, plants were then inoculated with TYLCV as described in the methods section. Samples of both the SA- and buffer- treated leaves were taken at 14 days after TYLCV inoculation. As a marker of the infection, we analyzed the accumulation of TYLCV by semi-quantitative PCR (semi-qPCR).

After two weeks of TYLCV inoculation, plants treated with buffer solution showed clear virus symptoms (e.g., yellowing and curly leaves), and there was a noticeable reduction in growth, while most of the SA-treated plants retained their original deep green color and exhibited no visible symptoms (Fig. 1A).

As Fig. 1B shows, all the SA- and buffer- treated leaves accumulated virus at 14 days after TYLCV inoculation. However, plants treated with SA had lower levels of viral contents when compared with buffer treated plants. TYLCV special fragments were detected after 32 and 26 cycles in SA and buffer-treated plants respectively. This results show that exogenous SA application inhibit virus multiplication in the plants.

After one month of TYLCV inoculation, disease incidence (%) and disease index were analyzed in SA and buffer treated plants. A significant difference in the disease incidence was observed in SA-treated plants with a value of 48.3%, compared to the buffer treated plants with a value of 76.4% (Fig. 1C). Similarly, disease index value was also lower for SA-treated plants (31.0) compared to that of buffer (45.1) (Fig. 1D). These results suggested that SA treatments induce resistance to TYLCV in tomato plants.

Supplemental Table 1. Primers used in the study A. Primer sequences used for the quantitative RT-PCR analysis of *DCL*, *AGO*, and *RDR* in tomato.

No.	Gene name (Accession no.)	Primers	Reference
1.	<i>SIDCL1</i> (Solyc 10g005130)	AAATGGGTTGTTAGTCGGTAT TTCAAAGCACCTTGTAATGC	this study
2.	<i>SIDCL2</i> (Solyc 06g048960)	GAGTGCCATAATGCACGAGG TTGCCAATAACTATCTGCTGTG	this study
3.	<i>SIDCL3</i> (Solyc 08g067210)	ATTTACCCAAGACAGTTTCG CCAGTTGCCTCCACATA	this study
4.	<i>SIDCL4</i> (Solyc 07g005030)	CTTTGTTGAACTACCTCCTG ATTGCTAACTCCCTCCC	this study
5.	<i>SIAGO1a</i> (Solyc 06g072300)	CTATCAGCCCCCAGTTACGTTTG ATCACCCCTTTTGACATTCTCCTTG	(Bai et al., 2012)
6.	<i>SIAGO1b</i> (Solyc 03g098280)	CAAGACACTGTTGCACATGGGTT CAAAAATGATTGTAGGACGGTCG	(Bai et al., 2012)
7.	<i>SIAGO10a</i> (Solyc 09g082830)	CGAGTTAGATGCAATTAGGAAGGC AATTAGTTTCAGGCATGTCTGGTTC	(Bai et al., 2012)
8.	<i>SIAGO5</i> (Solyc 06g074730)	TAGTAAGAACATGCCTTTCCTCACC GCAGCTCTTACATCCACGTCATTC	(Bai et al., 2012)
9.	<i>SIAGO7</i> (Solyc 01g010970)	GGCTGGCATGTTTCGAGATTTC GTGGCACCAGTGAAATAGGCTTG	(Bai et al., 2012)
10.	<i>SIAGO2a</i> (Solyc 02g069260)	TTGGCGAAGGCTATATACGACAG AGTTGCAGAGCTAGGAGAGTTCATC	(Bai et al., 2012)
11.	<i>SIAGO2b</i> (Solyc 02g069270)	ATCGTTACAAGTATAAACCTGAAATCAC GGCAGGTGAAGTTGTAGAGCTAGAA	(Bai et al., 2012)
12.	<i>SIAGO3</i> (Solyc 02g069280)	CTCCCTTGAGCCCATTTCCTT AAATCCTGGTCTTCACTTTGG	this study
13.	<i>SIAGO6</i> (Solyc 07g049500)	TCTCATCTACCGCTTATTAAGGACACT GAGATTTTGCAGGACATCAGGTG	(Bai et al., 2012)
14.	<i>SIAGO4a</i> (Solyc 01g008960)	TCCTGCTTTGTTTGCTTCCTGAG CCTCCCTCATTATTCCATCATCTTC	(Bai et al., 2012)
15.	<i>SIAGO4b</i> (Solyc 06g073540)	CTGTGGTCGACTTTCTGATAGCG ATTCCGGGTGAAAAGGTCATCT	(Bai et al., 2012)
16.	<i>SIAGO4c</i> (Solyc 06g073530)	CTGTTGAGGTTTCTCGTTCCATC CTGATGTGCCCTCGGATTTC	(Bai et al., 2012)
17.	<i>SIAGO4d</i> (Solyc 01g096750)	ACCTCAGATTTCCAAAGTCCCCA GCATGTCATCTGCTGAGAATCCAAT	(Bai et al., 2012)
18.	<i>SIAGO15</i> (Solyc 03g111760)	TCTCTTTAGACCAGTTTCAGATAGGG CATCTGAGCTGAAACCAAACGAG	(Bai et al., 2012)
19.	<i>SIRDR1</i> (Solyc 05g007510)	AAGACATGATCCCGCCAAGG ATGCCTTCATAATGCCACCACTAA	(Bai et al., 2012)
20.	<i>SIRDR2</i> (Solyc 03g114140)	TCAACCTCTTTCTCTGCTCCATAGTA TGGAGATGTAAGAGTACTTGAGGCTG	(Bai et al., 2012)
21.	<i>SIRDR6a</i> (Solyc 04g014870)	TCAAGTTCAACATACGGACATAATAGAT CATCATCTATGAAATCTTCGTATCCC	(Bai et al., 2012)
22.	<i>SIRDR6b</i> (Solyc 08g075820)	GCGAGGCTGAAGCAGGCATA GCGAGGTAGAGTCGGATTGTTG	(Bai et al., 2012)
23.	<i>SIRDR3a</i> (Solyc 12g008410)	TCATTGGAATAAATACACCCAGTCT CGAGGTTCTTCACAGGGACG	(Bai et al., 2012)
24.	<i>SIRDR3b</i> (Solyc 06g051170)	TCTTCTGACGCTGCGAGATGATA TGTTCAAACCTCCACAGCACCATA	(Bai et al., 2012)
25.	<i>SIEF1a</i> (X14449)	GACAGGCGTTCAGGTAAGG CCAATGGAGGGTATTCAGC	(Bai et al., 2012)

B. Primer sequences used for VIGS of *DCL* genes in tomato.

No.	Gene name	Primers
1	VIGS-SIDCL4-F (XbaI)	<u>GCTCTAGACAAGCCAAGGTCATAGAGG</u>
	VIGS-SIDCL4-R (KpnI)	<u>GGGGTACCAGATGCAGTCATGCCAAAT</u>
2	VIGS-SIDCL2a-F (XbaI)	<u>GCTCTAGACTGGCAAACATCCTCTTCA</u>
	VIGS-SIDCL2a-R (KpnI)	<u>GGGGTACCCAGCATCCCAATAATCAAC</u>

C. Primer sequences used for semi-quantitative PCR to detect TYLCV in tomato.

No.	Name	Primers	Reference
1.	TY-F/R(530-928)	ATTGGGCTGTTTCCATAGGGC CACACGGATGGGAAATACTT	
2.	<i>Actin</i> (TC198350)	GGAAAAGCTTGCCTATGTGG CCTGCAGCTTCCATACC	

D. Primer sequences used for RT-PCR to detect SA-mediated defense marker genes in tomato.

No.	Gene name	Primers	Reference
1.	<i>SIEF1a</i> (X14449)	GACAGGCGTTTCAGGTAAGG CCAATGGAGGGTATTCAGC	(Li <i>et al.</i> , 2013)
2.	<i>TYLCV-V1</i> (AB110218)	GAAGCGACCAGGCGATATAA GGAACATCAGGGCTTCGATA	(Sinisterra <i>et al.</i> , 2005)
3.	<i>SIPR1</i> (NM_001247429)	AACGCTCACAATGCAGCTCGT AAGGTCCACCAGAGTGTTGC	(Eybishtz <i>et al.</i> , 2010)
4.	<i>SIPR1b</i>	TTTCCTTTTGATGTTGCT TGGAACAAGAAGATGCAGT	(Kawazu <i>et al.</i> , 2012)

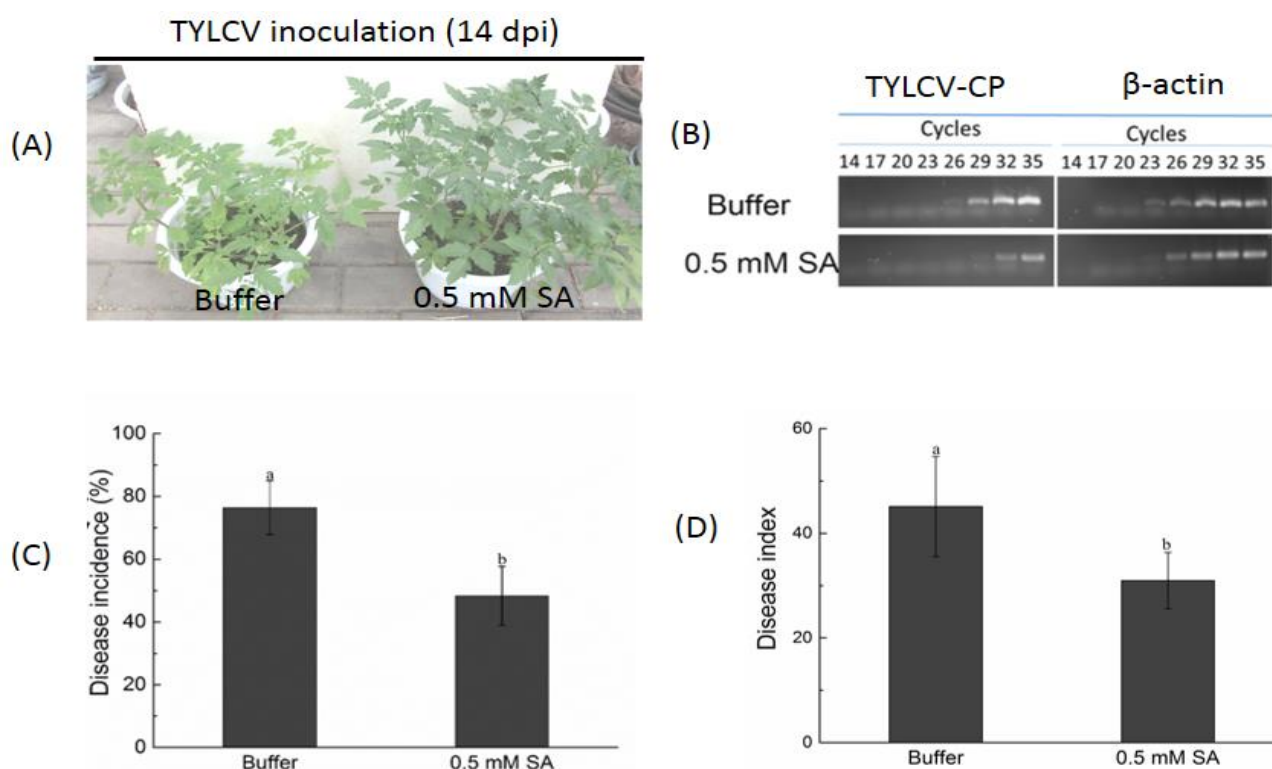


Fig. 1. SA-treated tomato plants. (A) Phenotype analysis of the SA (right) and buffer treated (left) tomato plants after 14 days of TYLCV inoculation. (B) TYLCV contents in SA (Left down) and buffer (left up) treated plants, by semi-qPCR at 14 days after TYLCV infection. (C) Disease incidence (%) and (D) Disease index were analyzed after one month of TYLCV inoculation. Values in (C-D) represent means \pm standard error (SE) of three replications. Different letters indicate significant difference between buffer and SA treated plants $p < 0.05$ using *t*-tests.

SA-induced RNA-silencing-related genes in tomato:

To explain increased resistance in SA-treated tomato plants against TYLCV, expression of RNA-silencing related genes families, such as the *SIDCL*, *SIAGO* and *SIRDR* families, were analyzed. The tomato pathogenesis-related *SIPR1* gene was used as a marker gene for the SA-treatment response (Fig. 2). The results showed that SA application significantly induced the expression of defense-related genes *SIPR1* and *SIPR1b* after 12 h compared to 0 h, which suggested that the application of exogenous SA triggered systemic resistance in the plants.

SIDCLs transcript abundance were analyzed by qRT-PCR. As shown in Fig. 3, transcript levels of *SIDCLs* were elevated in SA-treated plants relative to untreated plants (0h). After SA treatment, a rapid accumulation of *SIDCL2* transcripts were observed after 3 h (2.8-fold) with a maximum at 24 h (65-fold) and 2.7-fold at 72 h. The expression patterns of *SIDCL1* and *SIDCL4* were both similar to that of *SIDCL2*, initially increased and then decreased gradually. However, the expression levels of *SIDCL2* and *SIDCL4* different from that of *SIDCL1* with respect to the time point that exhibited the most variation, while *SIDCL3* expression did not vary substantially across different time periods.

Two other important core gene families, *RDRs* and *AGOs*, were examined for a potential role in the RNA-silencing mechanism. Figures 4 and 5 showed that SA treatments significantly induced the expression of some genes within the *SIRDRs* and *SIAGOs* families. Most of the genes exhibited maximum expression levels at 24 h following SA application. For *SIRDRs* genes, such as *SIRDR2*, *SIRDR3a*, *SIRDR6a*, and *SIRDR6b*, the expression levels substantially changed at 24 h after 0.5 mM SA application. In case of *SIRDR3b*, expression was highest 6 h after 0.5 mM SA treatment, while the expression level of *SIRDR1* remained stable from 12 h to 72 h (about 15-fold above baseline).

Additionally, most of *SIAGOs* genes were induced within 24 h after 0.5 mM SA treatment. The expression level of *SIAGO1* was highest at 24 h, followed by *SIAGO1b*, *SIAGO2a*, *SIAGO2b*, *SIAGO4a*, *SIAGO5* and *SIAGO15a*; the genes *SIAGO3*, *SIAGO4b*, *SIAGO4c*, *SIAGO4d*, *SIAGO6*, and *SIAGO7* had even lower

expression levels at this time. For *SIAGO1b* and *SIAGO15a*, the expression levels peaked at 12 h after SA treatment. These results showed that SA application clearly induced the expression of RNA-silencing related genes in tomato plants.

RNA-silencing-related genes induced by TYLCV: To confirm that *SIDCLs*, *SIRDRs*, and *SIAGOs* genes families participate in the plant antiviral response, we analyzed the relative expression of those genes following TYLCV inoculation by qRT-PCR.

The expressions of some RNA-silencing-related genes were significantly different between the TYLCV inoculated and non-inoculated (control) plants. As shown in Fig. 6A, the expressions of *SIDCL1*, *SIDCL2*, *SIDCL3* and *SIDCL4* were induced significantly in TYLCV inoculated plants, compared to that of control ones. Among the *SIDCLs* genes family, the expression of *SIDCL2* was highest (24-fold) followed by *SIDCL4* and *SIDCL1* with 10 and 7-folds respectively. On the other side, the expressions of these genes were remained lowered in the control plants.

Similar to *SIDCLs* family, all *SIRDRs* genes were up-regulated after TYLCV inoculation. As shown in Fig. 6B, five *SIRDRs* genes, i.e., *SIRDR1*, *SIRDR2*, *SIRDR3a*, *SIRDR6a*, and *SIRDR6b* were significantly up-regulated, especially *SIRDR2* (27-fold), followed by *SIRDR6b* (10.6-fold). *SIRDR1* showed the lowest expression level with a 4.5-fold increased.

SIAGOs is another important gene family for RNA-silencing. Not all *SIAGOs* genes were up-regulated after TYLCV inoculation. As shown in Fig. 6C, *SIAGO2a*, *SIAGO3*, and *SIAGO5* showed highly significant increase in expression, but *SIAGO3* was the most highly expressed than the control. Meanwhile, *SIAGO4c* and *SIAGO4d* were significantly repressed following viral inoculation. Other members of this family exhibited non-significant differences in expression level. Among the genes with induced expression, the greatest increased (48-fold) was observed for *SIAGO3*. The overall results showed that most of the RNA-silencing-related genes were induced after TYLCV inoculation, which described relation of these genes in TYLCV infection..

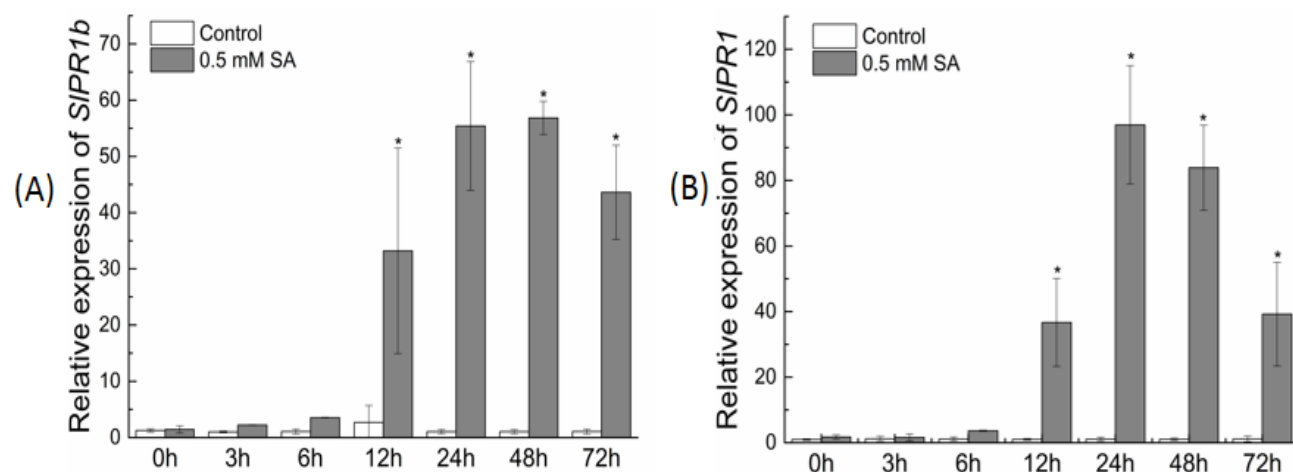


Fig. 2. Expression of the pathogenesis-related gene *SIPR1* and *SIPR1b* (a marker gene of SA-mediated defense) was induced by SA treatment. The relative expression of *SIPR1* and *SIPR1b* (two marker genes of SA-mediated defense) were determined by qRT-PCR. Values are means \pm standard error (SE), replicated thrice. The treatments were compared with the control. An asterisks means significant at $p < 0.05$ using *t*-test.

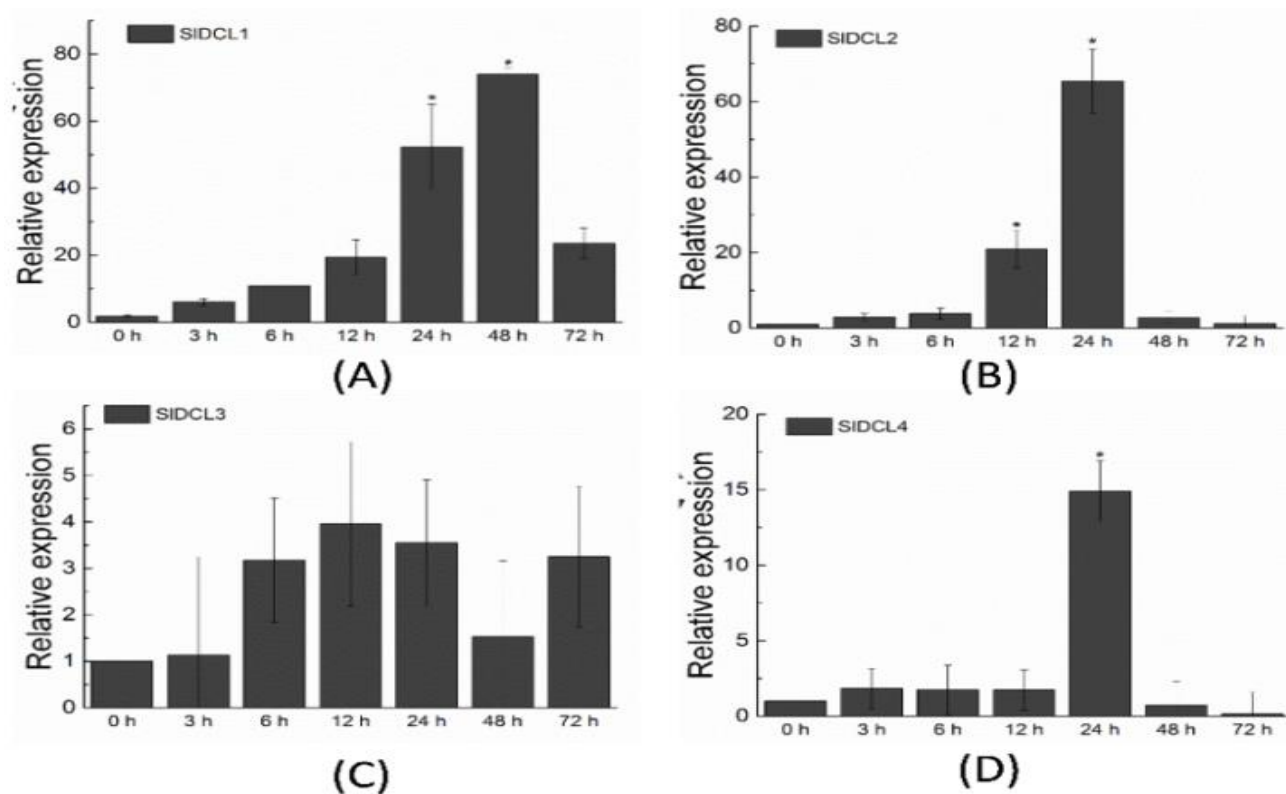


Fig. 3. Expression fold changes for *SIDCLs* after SA treatment at different times. The relative expression of *SIDCLs* were determined by qRT-PCR. Values are the mean \pm standard error (SE), replicated thrice. Values were first normalized to the *SIEF1a* expression level and are expressed relative to the mRNA level at 0 h. The treatments were compared with 0 h using *t*-test. An asterisks means significant at $p < 0.05$.

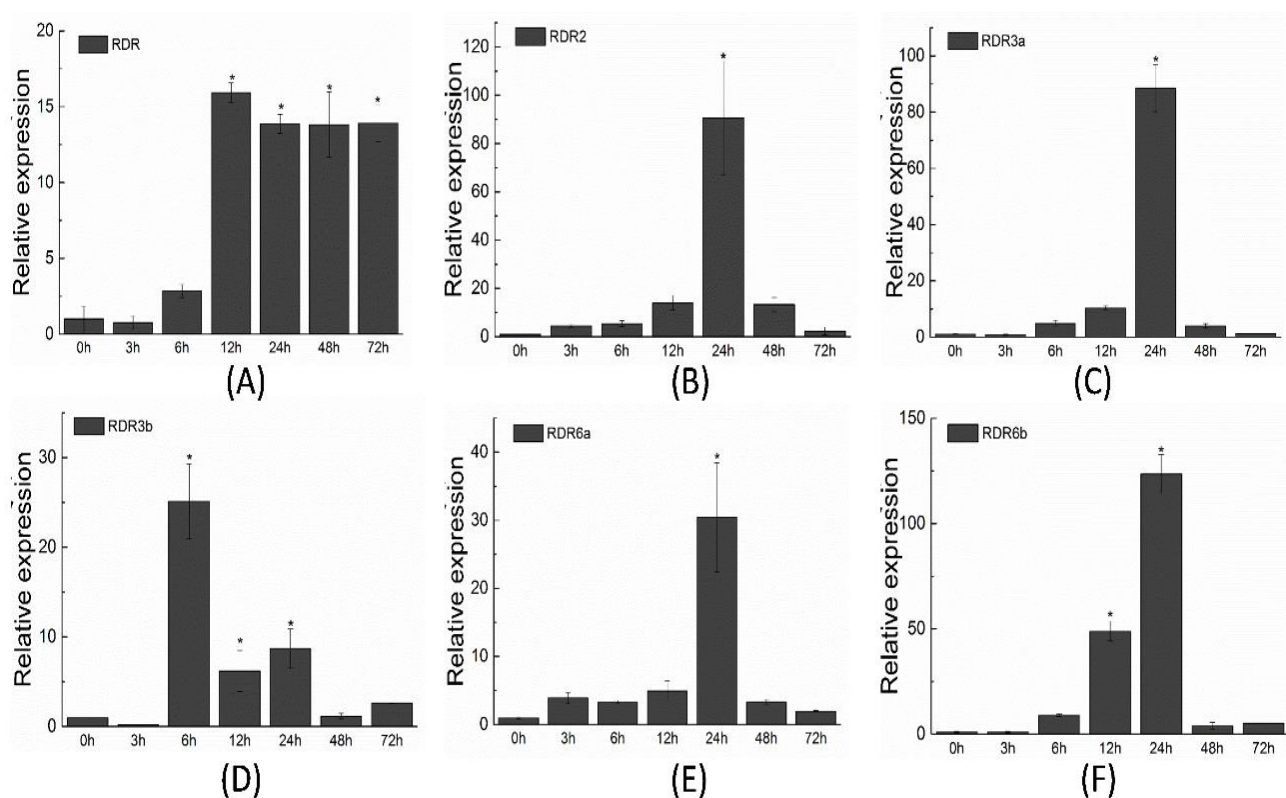


Fig. 4. Expression fold changes for *SIRDRs* after SA treatment at different times. The relative expression of *SIRDRs* were determined by qRT-PCR. Values are the mean \pm standard error (SE), replicated thrice. Values were first normalized to the *SIEF1a* expression level and are expressed relative to the mRNA level at 0 h. The treatments were compared with the 0 h using *t*-test. An asterisks means significant at $p < 0.05$.

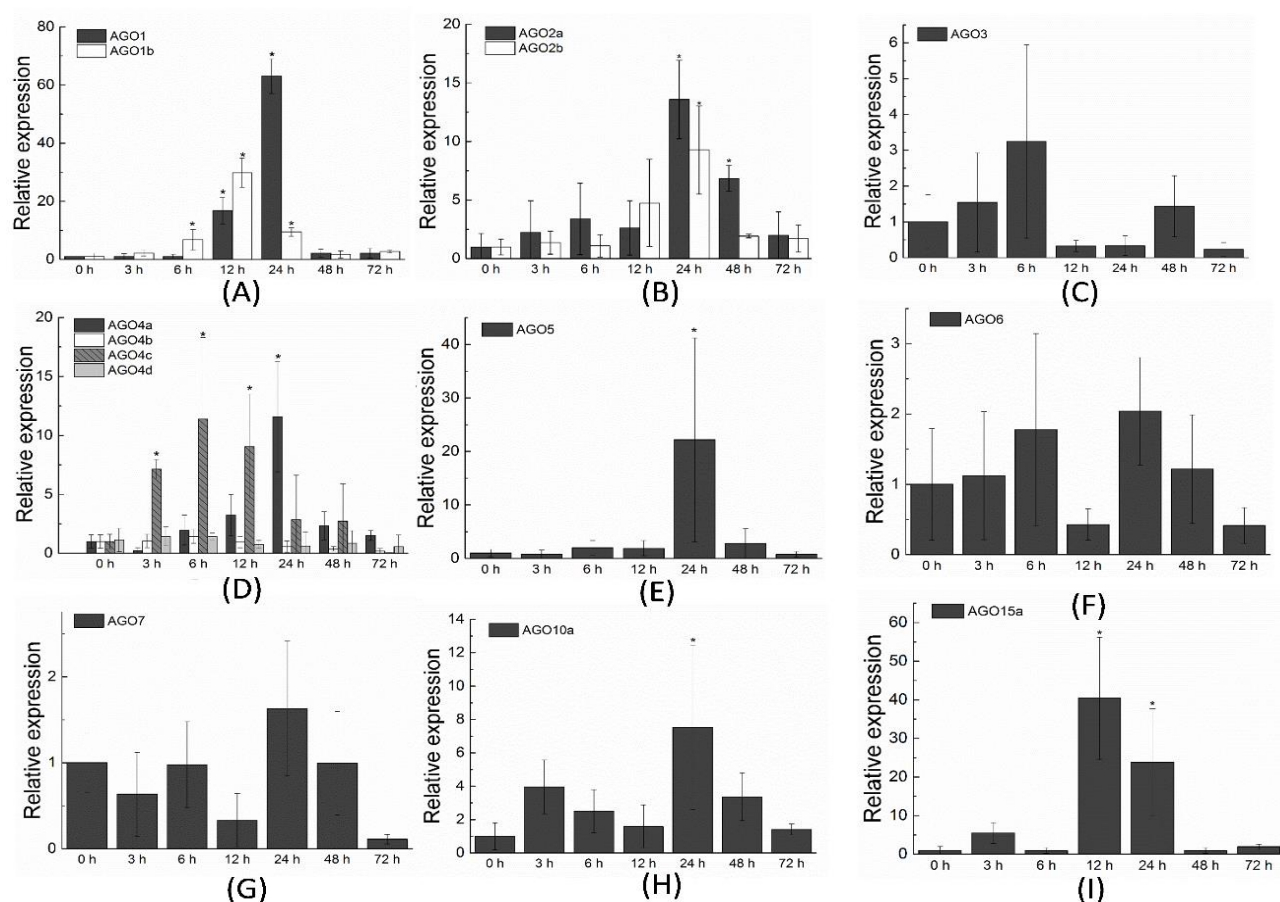


Fig. 5. Expression fold changes for *SIAGOs* after SA treatment at different times. The relative expression of *SIAGOs* were determined by qRT-PCR. Values are the mean \pm standard error (SE), replicated thrice. Values were first normalized to the *SIEF1a* expression level and are expressed relative to the mRNA level at 0 h. The treatments were compared with the 0 h using *t*-test. An asterisks means significant at $p < 0.05$.

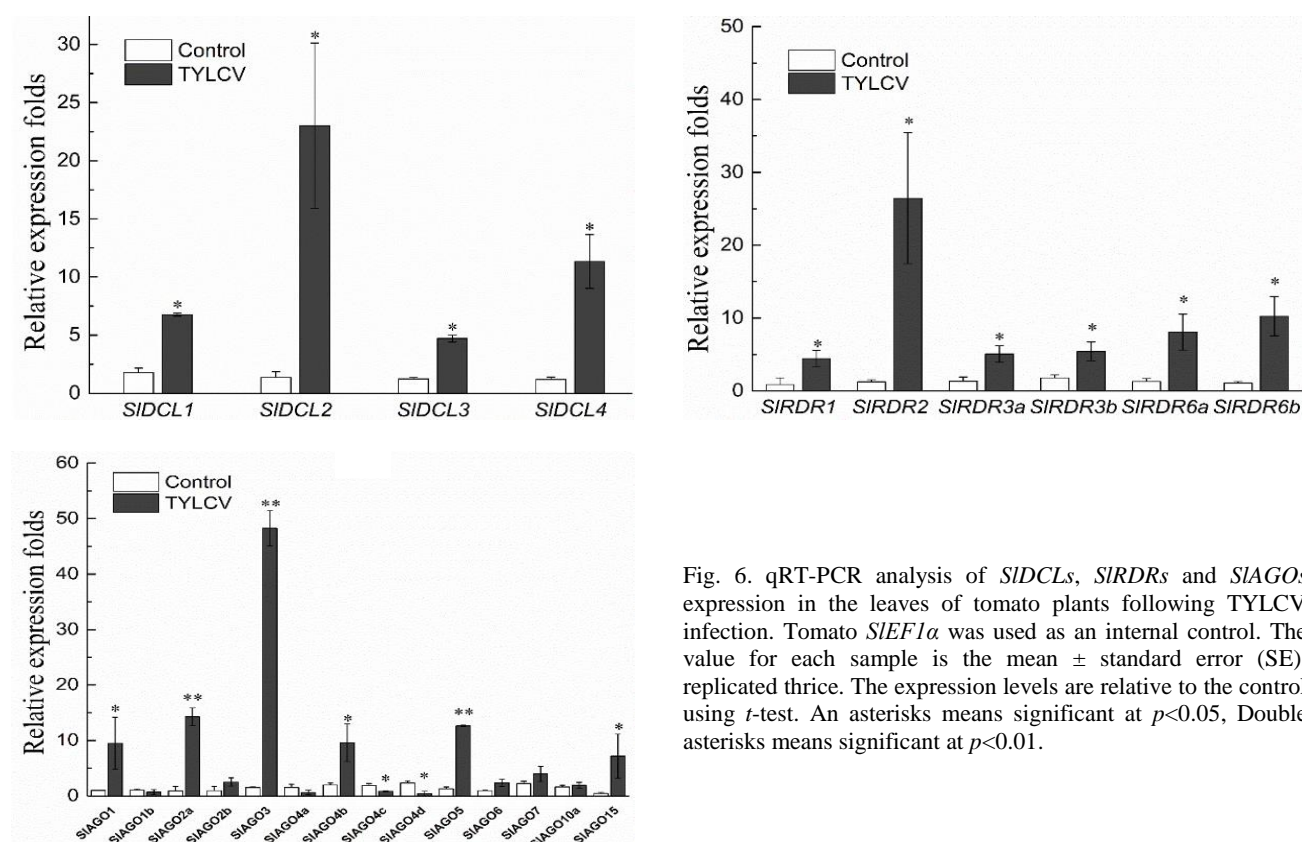


Fig. 6. qRT-PCR analysis of *SIDCLs*, *SIRDs* and *SIAGOs* expression in the leaves of tomato plants following TYLCV infection. Tomato *SIEF1a* was used as an internal control. The value for each sample is the mean \pm standard error (SE), replicated thrice. The expression levels are relative to the control using *t*-test. An asterisks means significant at $p < 0.05$, Double asterisks means significant at $p < 0.01$.

SIDCL2/SIDCL4-silenced tomato plants reduced resistance to TYLCV: Our above results show that *SIDCL2* and *SIDCL4* were up-regulated after TYLCV inoculation and SA application. In addition previous reports also described that, *AtDCL2* and *AtDCL4* are important genes which participate in RNA silencing triggered by RNA viruses (Deleris *et al.*, 2006). So, on the basis of our results and previous reports, we were interested in the characterization of *SIDCL2* and *SIDCL4*.

To confirm the function and importance of *SIDCL2* and *SIDCL4* in TYLCV infection, we silenced these two genes in tomato using the virus induced gene silencing (VIGS) method. When marker silencing plants (TRV: *PDS*) exhibited a photo-bleaching phenotype (Supplemental Fig. 1), we detected silencing efficiency from top leaves of *SIDCL2*, and *SIDCL4*, silenced plants (inoculated with TRV2-*SIDCL2* and TRV2-*SIDCL4*) and negative control (inoculated with TRV2). Compared to negative control, the *SIDCL2* and *SIDCL4* silencing efficiency reached about 75% and 55%, respectively (Supplemental Fig. 2) under normal conditions. At the same time, the other three genes of the family were not silenced (Supplemental Fig. 2). The results showed that VIGS was successful and effective for silencing *SIDCL2* and *SIDCL4* genes.

After 14 days of TYLCV inoculation, obvious yellowing and curling appeared in *SIDCL2* and *SIDCL4*-silenced plants, especially in *SIDCL2/4*-cosilenced plants, while control plants exhibited only slight disease symptoms (Fig. 7A).

In order to confirm the influence of *SIDCL2*- and *SIDCL4*- silencing in the antiviral defense response, the TYLCV contents were measured in silenced and non-silenced plants. Two weeks after TYLCV inoculation, the TYLCV contents were significantly higher in *SIDCL2*-, *SIDCL4*- and *SIDCL2/4*- silenced plants compared to control plants (Fig. 7B, C). Interestingly, *SIDCL2/4*-cosilenced plants were showed higher TYLCV content.

DCL2 and DCL4 are the enzymes responsible for the generation of virus-derived siRNAs (Molnar *et al.*, 2005; Garcia-Ruiz *et al.*, 2015). Their loss of function by mutation of both genes DCL2 and DCL4 is sufficient to make plants highly susceptible to several RNA viruses (Deleris *et al.*, 2006). To further confirm the functions of *SIDCL2* and *SIDCL4*, a similar experiment was performed using tomato material 'Y19' consisting resistance markers already described in materials and methods. After two weeks of TYLCV inoculation, top leaves of *SIDCL2*-, *SIDCL4* and *SIDCL2/4*-cosilenced plants exhibited stunting, upward leaf curling, and yellowing symptoms, prominently in *SIDCL2/4*-cosilenced plants (Fig. 8A). To further verify the results, TYLCV contents were checked and found that contents were also higher in *SIDCL2*-, *SIDCL4*- and *SIDCL2/4*-silenced plants than in the control plants, particularly in *SIDCL2/4*-cosilenced plants (Fig. 8B, C). These results confirmed that silencing of both *SIDCL2* and *SIDCL4* increased susceptibility to TYLCV, and suggested that these two genes should be considered core components in the RNA silencing pathway against DNA viruses.

Silencing of both SIDCL2 and SIDCL4 significantly decreased the expression of SA signaling-regulated defense genes upon infection of TYLCV in tomato plants: To investigate the relationship between RNAi and SA-mediated defense against TYLCV, we analyzed the expression of representative marker genes regulated by the SA-mediated defense signaling pathway to explore the molecular mechanism associated with the reduced TYLCV resistance in *SIDCL2*- and *SIDCL4*-silenced plants. Researchers reported two marker genes, *SIPR1* and *SIPR1b*, which are regulated by SA-mediated signaling pathway (Kawazu *et al.*, 2016). The expression levels of *SIPR1* and *SIPR1b* were compared in *SIDCL2*-, *SIDCL4*-, *SIDCL2/4*-silenced and control plants. No significant difference was observed in the expression of these two genes between *SIDCL2*-, *SIDCL4*-, *SIDCL2/4*-, and control plants in normal conditions (Fig. 9), indicating that the silencing of *SIDCL2*, *SIDCL4*, and *SIDCL2/4* did not affect the expression of *SIPR1* and *SIPR1b* in normal tomato plants.

After TYLCV inoculation, the expression levels of *SIPR1* and *SIPR1b* increased; however, the expression levels in TRV: 00 plants were significantly higher than those in gene-silenced plants. In *SIDCL2*- and *SIDCL4*-silenced plants, *SIPR1* and *SIPR1b* were highly expressed than in *SIDCL2/4*-cosilenced plants after TYLCV inoculation (Fig. 9A-B). These results indicate that both *SIDCL2*- and *SIDCL4*-silencing plants unable to follow SA signaling-regulated defense genes pathway in response to TYLCV infection, which further verify the contributing role of RNAi and SA defense pathways against DNA viruses.

Discussion

This study initially confirmed that foliar application of SA, a promising non-transgenic strategy, enhanced resistance to TYLCV in tomato plants, which is in consistent with a previous study that SA accumulates after TYLCV infection (Sade *et al.*, 2014). Our results are also consistent with those studies for PVX infections and other RNA viruses (Shang *et al.*, 2011; Falcioni *et al.*, 2014). The reason that the disease incidence was lower in SA-treated plants might be because SA plays a crucial role in host-virus interactions. Some studies also reported that SA treatment enhanced the ability of antiviral activity in plants or delayed the appearance of disease symptoms because of SAR (Shang *et al.*, 2011; Campos *et al.*, 2014). In plants, SAR is biologically induced by localized infections with pathogens or is linked with systemic accumulation of SA and certain pathogenesis-related (PR) proteins (Hao *et al.*, 2015). Moreover, increases in SA levels have been observed in inoculated and non-inoculated systemic tissue before the establishment of induced resistance (IR) (Vlot *et al.*, 2009). Similarly in NahG transgenic plants, the SA-degrading enzyme salicylate hydroxylase reduces SA production, resulting in increased disease susceptibility and an inability to respond via SAR after biological induction (Benouaret & Goupil, 2015).

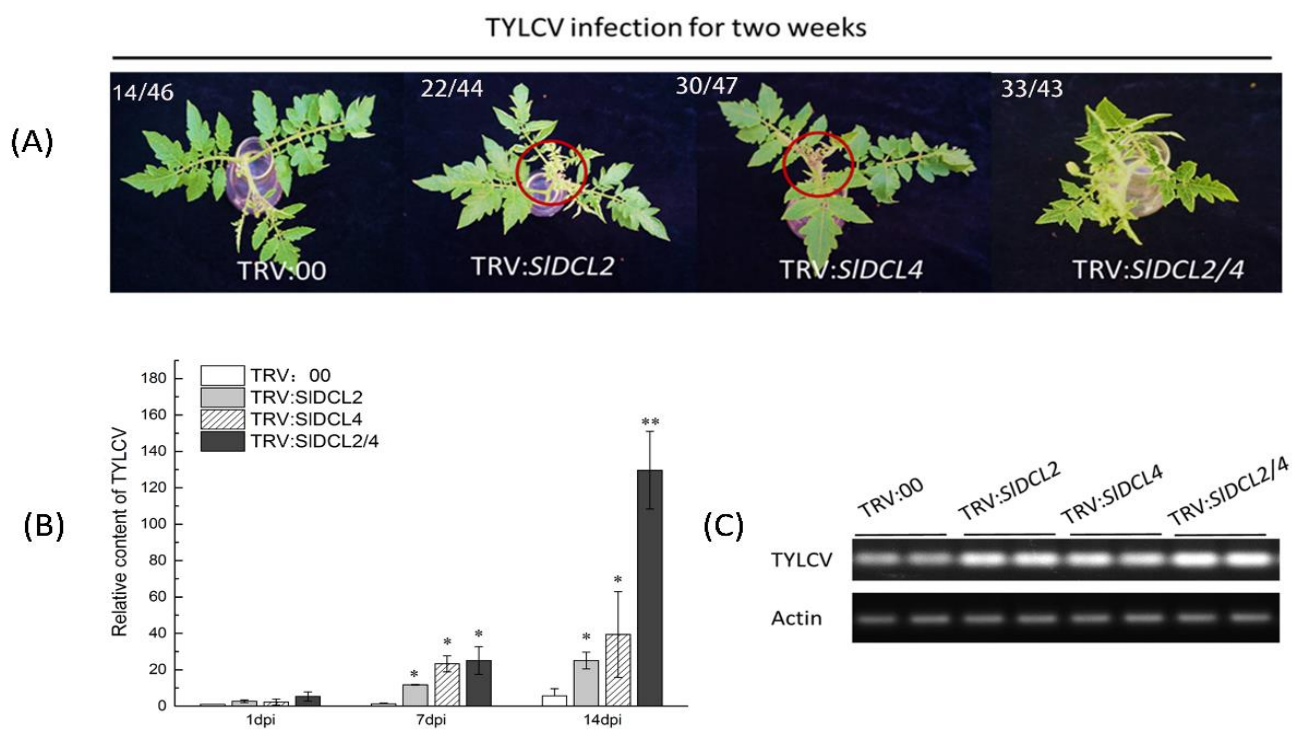


Fig. 7. *SIDCL2*, *SIDCL4* and *SIDCL2/4*-silenced tomato plants. (A) Phenotype analysis of the *SIDCL2*, *SIDCL4* and *SIDCL2/4*-silenced and control tomato seedlings after TYLCV infection for 14 days. (B) The content of TYLCV in gene-silenced tomato (TRV2: *SIDCL2*, TRV2: *SIDCL4* and TRV2: *SIDCL2/4*) and control plants (TRV2:00) were tested by qPCR at 7 and 14 days after TYLCV infection. Relative TYLCV content are relative to the control using *t*-test. An asterisks means significant at $p < 0.05$, Double asterisks means significant at $p < 0.01$. (C) The content of TYLCV in gene-silenced tomato (TRV2: *SIDCL2*, TRV2: *SIDCL4* and TRV2: *SIDCL2/4*) and control plants (TRV2:00) were tested by semi-qPCR at 14 days after TYLCV infection.

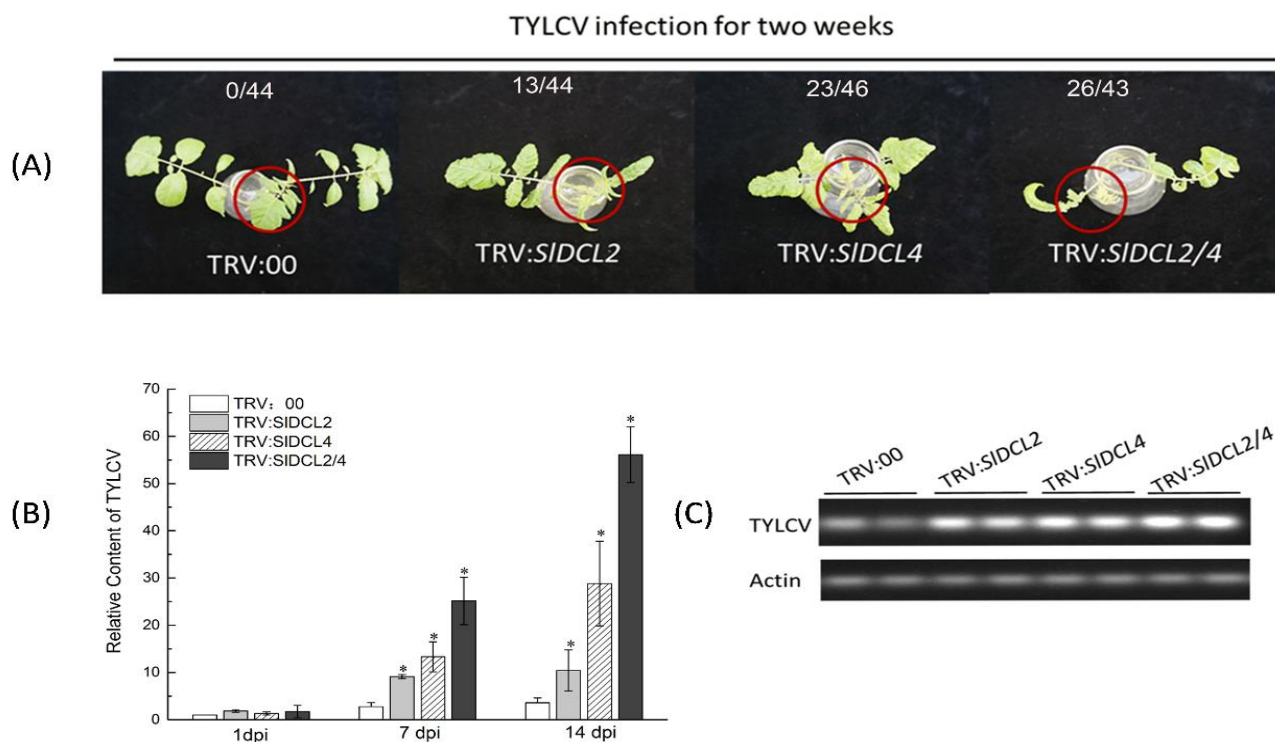


Fig. 8. *SIDCL2*, *SIDCL4* and *SIDCL2/4*-silenced 'Y19' tomato plants with resistance marker. (A) Phenotypic analysis of TRV: 00 (control), TRV: *SIDCL2*, TRV: *SIDCL4* and TRV: *SIDCL2/4*-silenced tomato plants after 14 days of TYLCV inoculation. The numbers above figure indicate disease symptoms producing plants and total number of plants. (B) TYLCV contents in TRV:00 (control), TRV:*SIDCL2*, TRV:*SIDCL4* and TRV:*SIDCL2/4*-silenced plants using qPCR at 1, 7 and 14 days after TYLCV inoculation. Relative TYLCV content are relative to the control using *t*-test. An asterisks means significant at $p < 0.05$. (C) TYLCV contents in TRV: 00 (control), TRV: *SIDCL2*, TRV: *SIDCL4* and TRV: *SIDCL2/4*-silenced plants using semi-qPCR at 14 days after TYLCV inoculation.

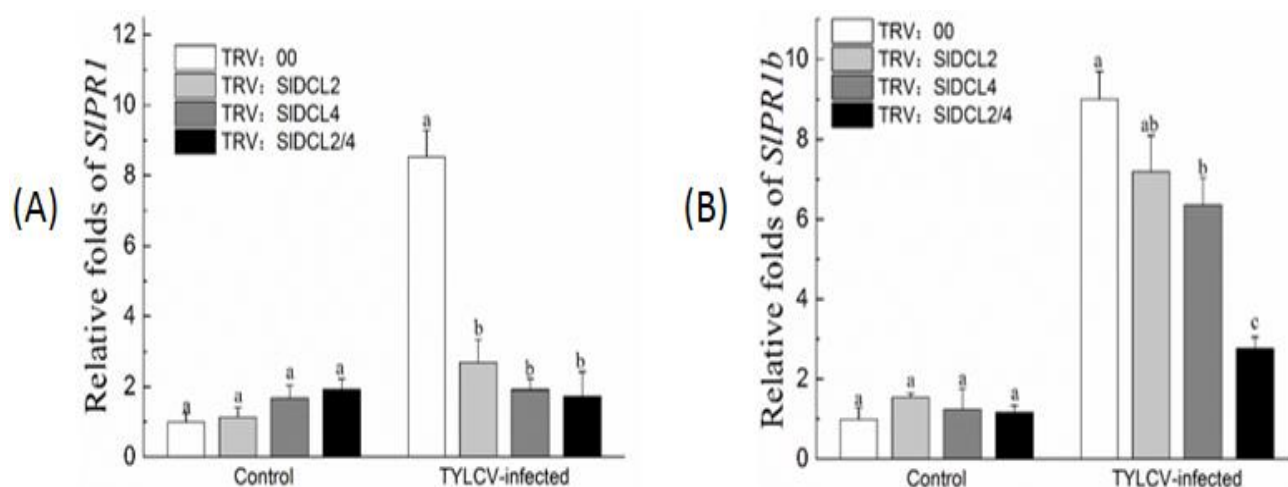


Fig. 9. qRT-PCR analysis of defense-related genes (*SIPR1* and *SIPR1b*) expression in gene-silenced tomato (TRV2: *SIDCL2*, TRV2: *SIDCL4* and TRV2: *SIDCL2/4*) and control plants (TRV2:00) after TYLCV infection for 24 h. (A) The Expression of *SIPR1* in gene-silenced tomato (TRV2: *SIDCL2*, TRV2: *SIDCL4* and TRV2: *SIDCL2/4*) and control plants (TRV2:00) pre- and post-TYLCV inoculation. (B) The Expression of *SIPR1b* in gene-silenced tomato (TRV2: *SIDCL2*, TRV2: *SIDCL4* and TRV2: *SIDCL2/4*) and control plants (TRV2:00) pre- and post-TYLCV inoculation. Tomato *SIEF1a* was used as an internal control. The value for each sample is the mean \pm standard error (SE), replicated thrice. The expression levels are relative to the control using *t*-test. Different letters above the columns indicate significant differences at $p < 0.05$.

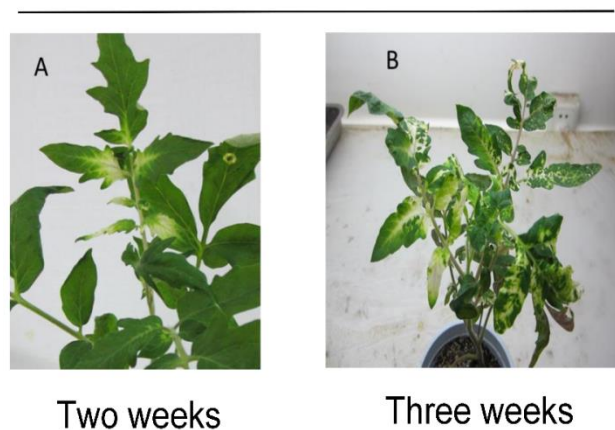
SIDCL2 and *SIDCL4* were confirmed to play a key role against TYLCV, using VIGS-silencing technology. We found that silencing of *SIDCL2* and *SIDCL4* resulted in a higher level of viral infection as compared to control plants and were accompanied by decreased expression of defence-related genes in SA-mediated pathways. The sensitivity to the virus was increased in co-silenced *SIDCL2/4* plants probably because *DCL2* and *DCL4* have key roles in antiviral RNA silencing. However, many reports have demonstrated that both *DCL2* and *DCL4* are related to activity against RNA viruses, such as TuMV, TRV, TCV, and CMV (Deleris *et al.*, 2006; Shang *et al.*, 2011; Garcia-Ruiz *et al.*, 2015). Our results are further supported by the fact that *dcl2* and *dcl4* (*DCL2* and *DCL4*) single mutants produce a similar degree of PVX systemic infection, but to a lesser extent than *dcl2* and *dcl4* double mutants (Brosseau & Moffett, 2015), and *DCL2* also plays an important role in RNA-silencing (Mlotshwa *et al.*, 2008). Silencing of the genes *DCL2/DCL4* enhanced *Citrus tristeza virus* (CTV) spread and accumulation in sour orange plants in comparison with non-silenced controls (Gómez-Muñoz *et al.*, 2016). In *Arabidopsis*, AtDCL2 and AtDCL4 processed dsRNA molecules into 22-nt and 21-nt siRNAs respectively, and both these siRNAs are required for optimal resistance against viruses (Parent *et al.*, 2015). In this study, tomato *SIDCL2* and *SIDCL4* are confirmed as two key genes in the RNA silencing mechanism, and this mechanism participates in antiviral defense against TYLCV. Furthermore, the relationship of RNA silencing and SA-mediated defence was studied via detection of two defense genes *SIPR1* and *SIPR1b*, which are marker genes in the SA-mediated pathway (Kawazu *et al.*, 2016). The expression of *SIPR1* and *SIPR1b* had not significantly changed in the *SIDCL2*-, *SIDCL4*-, *SIDCL2/4*-, and empty vector-silenced plants without TYLCV inoculation. However, the expression of *SIPR1* and *SIPR1b* was significantly lower in

SIDCL2/4-silenced plants than in *SIDCL2*-, *SIDCL4*-, and empty vector-silenced plants after TYLCV infection, indicating that *SIDCL2* and *SIDCL4* may be involved in SA-mediated signaling pathways in tomato plants upon TYLCV infection. *DCL2* and *DCL4* are required for the production of “primary” siRNA (Moissiard *et al.*, 2016). It is possible that siRNA could also target some unknown genes which regulated the expression of defense or SA-mediated defense. Other interesting genes related to RNA silencing are *RDRs*, these genes are reported to be involved in production of “primary” siRNAs and “secondary” siRNAs (Lee *et al.*, 2016; Moissiard *et al.*, 2016). In this study, the expression levels of *SIRDR3a* and *SIRDR6a* were induced by both SA application and TYLCV infection. Other *RDRs*, like *SIRDR2* and *SIRDR6b*, were induced only by SA application, and *SIRDR1* participated in response to TYLCV infection. Similarly, *RDR1* is also induced by virus infections in tobacco and *Arabidopsis* (Ji *et al.*, 2009; Liao *et al.*, 2015). More importantly, it was reported that *RDR1* was induced by SA and participated in the antiviral response (Lee *et al.*, 2016), which confirmed that *RDR1* have an important role in antiviral activity. In addition, *RDR6* plays an important role in antiviral and signal amplification of RNA silencing, supported by the finding that *RDR6* shows high expression levels after virus infection in tomato (Campos *et al.*, 2014). Recently two TYLCV tolerance genes (*Ty-1* and *Ty-3*) were cloned and it was suggested that they are allelic encoding an *RDR*, which belonging to the *RDRγ* type, and are involved in the amplification of siRNA signals (Verlaan *et al.*, 2013). However, *SIDCL2* and *SIDCL4* silencing also lowered defense against TYLCV in tomato material carrying the *Ty-1* and *Ty-3* marker genes (Fig. 8), which suggested that *DCL2* and *DCL4* processing of “primary” siRNA is also important (Moissiard *et al.*, 2016). As the production of “secondary” siRNA by *RDR6* needs the triggering of

“primary” siRNA (Moissiard *et al.*, 2016), so the activities of DCL4 and DCL2 are necessary to generate secondary siRNAs downstream from RDR6 action (Moissiard *et al.*, 2016). This was the reason that silencing of *SIDCL2* and *SIDCL4* reduced resistance to TYLCV in ‘Y19’ tomato material with *Ty-1* and *Ty-3* resistance markers (Fig. 8). In TYLCV-infected tomato plants, we observed highly induced expression of many *SIAGO*s genes, with the exception of *SIAGO4c* and *SIAGO4d*. The *SIAGO2a*, *SIAGO3*, and *SIAGO5* were the most highly induced genes. *AGO5* plays an essential role in limiting PVX infection in systemic tissues in *Arabidopsis* (Brosseau & Moffett, 2015), consistent with the high expression of *SIAGO5* genes after TYLCV inoculation. *AGO2* antiviral activity has been demonstrated for several viruses and is more important than *AGO1* in defense against wild-type TCV (Zhang *et al.*, 2012). In addition, *AGO2* regulates microRNA (miRNA) activities by selective autophagy degradation (Gibbings *et al.*, 2015). Increased *SIAGO2* and *SIAGO1* expression was also observed after virus infection because they exhibited an important function in transcriptional silencing (Janowski *et al.*, 2006).

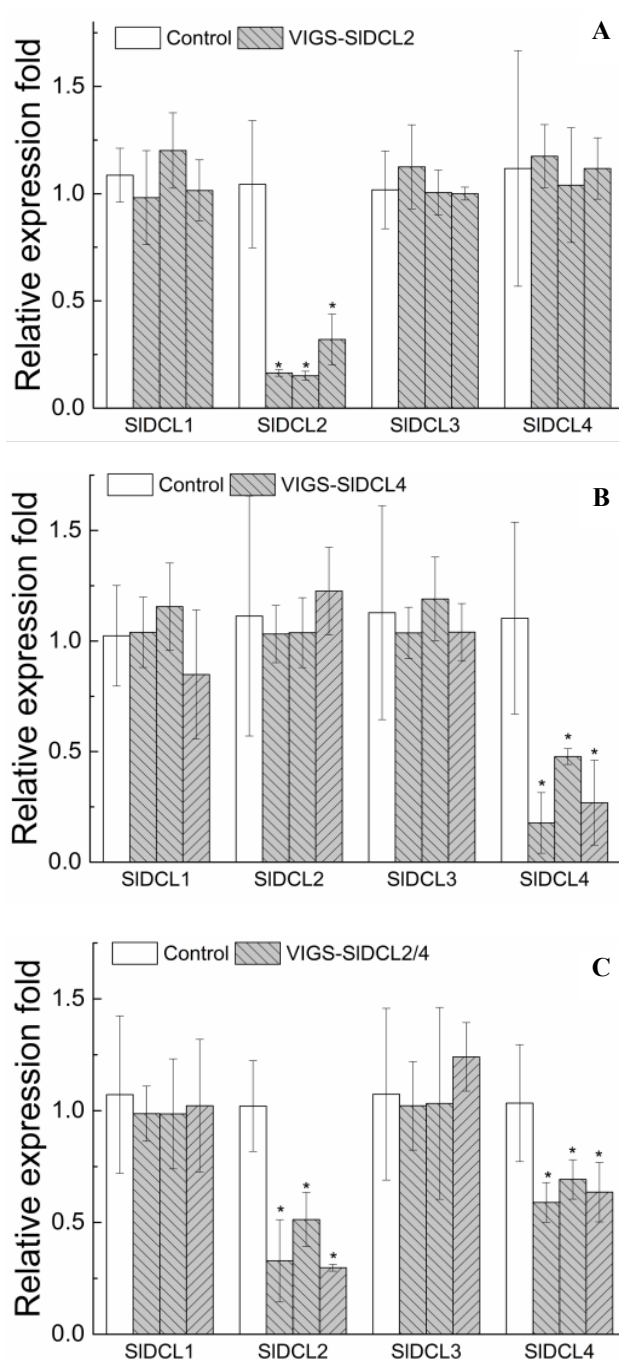
Interestingly, *SIAGO4c* and *SIAGO4d* were found to be down-regulated after virus inoculation and these effects were accompanied by a reduction in methylation directed by *AGO4* (Gao *et al.*, 2010). The reduction in *AGO4*-mediated methylation after TYLCV infection might be explained by repression during specific vegetative and reproductive developmental stages. *Ago4* is associated with RNA-directed DNA methylation (Duan *et al.*, 2015). There is a possibility that DNA methylation also participates in antiviral responses. Further in-depth study would be required to confirm this.

TRV:PDS



Supplemental Fig. 1. Silencing of the tomato *PDS* genes as a marker for the efficiency of VIGS silencing. Plants infected with TRV carrying the tomato gene for *PDS*. Silencing of the endogenous plant *PDS* causes the inhibition of carotenoid biosynthesis and results in a photo-bleaching phenotype.

Fig. S1 A. *PDS*-silenced plants after VIGS inoculation at 14 dpi. B. *PDS*-silenced plants after VIGS inoculation at 21 dpi. Photographs were taken 2 and 3 weeks after TRV infiltration, respectively.



Supplemental Fig. 2. Silencing efficiency and specificity for *SIDCL2* and *SIDCL4*

Tomato *SIEF1a* was used as an internal control for qRT-PCR and fold changes indicate expression levels in treated leaves relative to expression levels for the negative control, which was set to 1. Mean values and SE were obtained from three technical and biological replicates.

Conclusion

SA application delayed viral disease symptoms and induced resistance to TYLCV. SA application also induced most of RNA-silencing-related genes, which are naturally triggered after virus infection in tomato plants. *SIDCL2* and *SIDCL4* silencing decreased the defense response and increased susceptibility to the virus, furthermore, SA-mediated defense genes (i.e.,

SIPRI and *SIPRIb*) were not induced in silenced plants, especially for *SIDCL2/4*-cosilenced plants. From all these results we concluded that RNA silencing, where *DCL2* and *DCL4* are the main DCL endoribonucleases, are important in SA-induced antiviral defense.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (2016YFD0101703) and the Science and Technology Innovation Project of Shaanxi Province (2015KTTSNY03-01). The infectious TYLCV clone was kindly provided by Professor Xueping Zhou (Zhejiang University). Many thanks to Dr. Libo Xing for primer synthesis and providing the plasmid extraction reagent. We thank Dr. Chunhui Song, Dr. Yin Zheng, Yanqing Yang, Dr. Wei Chen, Tuo Qi, and Dr. Shuang Song for advice. We thank Wei Chen for the help with agro-infiltrations. Thanks are also due to Dr. Yan Zhang, Dr. Hehe Cao, Leonie Lin and Mogo Internet Technology Company (www.mogoeedit.com) for polishing the manuscript.

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(Received for publication 19 October 2017)